Consommation et Corporations Canada | Consumer and Corporate Affairs Canada
Bureau des brevets | Patent Office
Ottawa, Canada K1A 0C9

(21) (A1) 2,028,968
(22) 1990/10/31
(43) 1991/05/25
(52) C.R. CL. 195-35
195-44
150-15

(51) INTL.CL.[5] C12N-011/00; C12N-001/00; C12Q-001/26; C12Q-001/44

(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Stabilized Dispersed Enzymes

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(30) (US) 07/440,697 1989/11/24

(57) 28 Claims

S1000-73

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Notice: The specification contained herein as filed

Canada

## ABSTRACT OF THE DISCLOSURE

Enzymes are immobilized, stabilized and evenly dispersed without destroying their activity by sequestering them within micropores of a solid carrier structure and filling the pores with a water-immiscible organic liquid, in which the enzyme remains active. The preferred solid carrier is a microporous membrane. The system is manufactured by providing a solid carrier having a microporous surface whose pores contain an aqueous solution, allowing an aqueous solution of enzyme to equilibrate with the solution in the pores, causing a controlled partial collpase of the pores to effectively trap the enzymes, and replacing the liquid in the pores with a water-immiscible organic liquid. The system can be used to assay for or produce an organic compound. It also can be used for slow release of an enzyme.

STABILIZED DISPERSED ENZYMES

Attorney Docket No. KTI-108A

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

This invention relates to enzymatic processes, e.g., for assaying or synthesizing specific organic compounds.

Enzymatic reactions are widely used in various biomedical and industrial applications such as assaying or synthesizing specific organic compounds. To facilitate enzyme handling and recovery, enzymes have been immobilized on insoluble supports, including membranes. Techniques for immobilization include chemical cross-linking, covalent binding to supports, physical entrapment or adsorption, or a combination of physical and chemical processes.

To be functional, enzymes must retain their natu-

rally occuring folding pattern which establishes the configuration or structure necessary for enzymatic activity. Denaturization of the enzyme (alteration of its folding pattern accompanied by loss of activity) can result from prolonged storage, heat, and other environmental factors. Many immobilization methods, particularly covalent binding and cross-linking, have been proposed to preserve the native conformation of the enzymes, but such methods themselves may contribute to enzyme inactivation.

Kazandjian _et al._, _Biotech. and Bioeng._ XXVIII:417-421 (1986) disclose a method of precipitating two enzymes, horseradish peroxidase and cholesterol oxidase, onto a glass powder. First they form an aqueous slurry of enzyme and powder, and then they dry the slurry to obtain "visibly dry (free-flowing) beads." They add the resulting beads to enzyme substrate (p-amisidine)

dissolved in various solvents, and conclude that the reaction proceeds fastest in

> "very hydrophobic, water immiscible solvents that evidently do not strip the essential water from the enzyme even if no exogenous water is added (on top of that brought in with $H_2O_2$) . . . . [Even] in toluene and other highly hydrophobic organic solvents, a certain amount of water present is required."

They attribute loss of enzymatic activity in less hydrophobic, more water-miscible solvents to stripping of critical water molecules from the enzyme.

Klibanov, <u>Science</u> <u>219</u>: 722-727 (1983) discloses various strategies for enzyme stabilization, including attaching the enzyme to a support by multiple links to avoid unfolding, and encapsulating the enzyme in membranes that are impermeable for enzymes, but permeable for low molecular weight substrates and products. Entrapment in microcapsules is accomplished by "interfacial polymerization, liquid drying or phase separation."

Entrapment in liposomes or in hollow fibers is also disclosed.

Zaks and Klibanov, Science, 224:1249-1251 (1984) and Zaks and Klibanov, Proc. Nat'l Acad. Sci. 82:3192-3196 (1985) disclose that porcine pancreatic lipase, yeast lipase, and mold lipase retain their activity in nearly anhydrous organic solvents. They further disclose that, while water is essential for maintenance of activity of these enzymes, it also participates in inactivation processes, particularly thermal inactivation. They further disclose that enzymes are more heat-stable in organic, water-immiscible solvents.

## SUMMARY OF THE INVENTION

I have discovered that enzymes can be stabilized and evenly dispersed without destroying their activity by sequestering the enzyme at least at the surface of

a solid carrier structure having micropores and filling the pores with a water-immiscible organic liquid, in which the enzyme remains active. The preferred solid carrier is a microporous membrane.

## BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is more fully understood when the specification herein is taken in conjunction with the appended drawings, wherein:

Figure 1 is a flow diagram of the steps for manufacturing an enzyme system.

Figure 2 is a graph demonstrating the system of example 1.

Figure 3 is a graph demonstrating the system of example 2.

Figure 4 is a graph demonstrating the system of

DETAILED DESCRIPTION OF THE PRESENT INVENTION

This invention teaches a novel, simple and practical way of achieving a uniform dispersion of one or more enzymes in a pre-formed polymer matrix such as a membrane and stabilization of such uniformly dispersed enzymes by essentially non-polar solvents as described by Kazandjian et al (supra) or Zaks et al (supra).

The method of achieving such a uniform dispersion falls in a class of methods of enzyme immobilization described as enzyme entrapment but differs from conventional entrapment methods in that it involves no cross-linking or polymerization reaction and thereby offers more flexibility in the choice of membrane materials and the enzymes themselves. Additionally, this method offers a way to stabilize the enzymes subsequent to entrapment.

The current state of art of immobilization of enzymes is summarized by Klibanov, (supra). As described therein, they can be divided into five classes:

a.) Covalent attachment; b.) Adsorption of enzymes on solid supports, typically ion exchange supports; c.) Entrapment of enzymes in polymeric gels; d.) Cross-linking of enzymes with trifunctional reagents; and, e.) Encapsulaton of enzymes.

The intent behind immobilization is usually to stabilize the enzymes against denaturation.

A common mechanism of denaturation is when the enzymes (which are essentially high molecular weight proteins with catalytic activity) lose their catalytic capability by losing their three dimensional strucure via chain unfolding which is responsible for its catalytic

action. The intent of immobilization is to restrict the movement of enzyme chains by securing at least a portion of enzyme chains to a support and/or by confining them in cage-like structures on a molecular scale such that they are much more restricted in their movements and the denaturation is slowed down or in other words enzymes are stabilized.

In many methods however such goals are not always realized. Particularly the methods that rely on covalent attachment, conventional entrapment and cross-linking with bifunctional reagents employ chemical reactions and often many enzymes don't survive the treatment. A large fraction of enzyme activity could be lost during the treatment itself. For each enzymes, for each type of reaction, and for each support usually extensive optimization need to be done.

The above methods and the physical adsorption methods additionally are generally unsuitable for applications which invovle multiple enzymes. Many diagnostics applications e.g. cholesterol, triglycerides, etc. use a one cascade of enzymes where product of one enzymatic reactions are a substrate for the next. When the enzymes are bound as in the above methods they usually will not couple with each other easily and the ration of various enzymes necessary for proper performance can also not be adjusted easily without extensive optimization experiments.

Encapsulation methods although useful for multiple enzymes also have some drawbacks that during encapsulation, the enzymes do come in contact with the organic solvents which often harmful to the enzymes.

In preferred embodiments, the organic liquid is characterized by a dipole moment less than 2.5 Debye

units, a dielectric constant less than 20, and a boiling point at atmospheric pressure of at least about 40°C but preferably over about 75°C. The system is particularly useful for enzymes such as cholesterol oxidase, whose substrates are insoluble or sparingly soluble (less than about 10g/l) in water under physiological conditions. Preferably the liquid content of the carrier is at least 90 percent water-immiscible organic liquid.

A second aspect of the invention generally features a method of making a stabilized, evenly dispersed enzyme system, by providing a solid carrier having a microporous structure whose pores contain an aqueous solution, allowing an aqueous solution of enzyme to equilibrate with the solution in the pores, and replacing the liquid in the pores with a water-immiscible organic liquid. Preferable, after the equilibration of the enzyme pores

are subjected to controlled partial collapse. By partial collapse, I mean that the pores are not eliminated, but they are collapsed sufficiently to effectively trap the enzyme. The preferred method of effecting controlled partial collapse and replacement of the aqueous liquid is to dry the carrier surface to remove water, introduce a water miscible organic liquid into the pores, and then contact the carrier with the immiscible organic liquid, allowing replacement by diffusion. Preferably, the largest membrane pores initially have a diameter in the range of 10 Angstroms to 100 microns. After controlled collapse, the pore size is reduced to sequester the enzyme (or cell debris containing the enzyme) to on the order of between 5 Angstroms and 10 microns.

The invention offers improved enzyme stability, e.g., as manifested by shelf-life in a diagnostic kit;

moreover it substantially improves heat tolerance of the enzyme, e.g. so that its catalytic reaction can be performed at temperatures well above ordinary physiological temperatures. Also, the invention provides an even dispersion of enzyme without formation of clumps or precipitation, which limit surface area contact with the reaction medium. The system is produced under mild conditions so as to preserve enzymatic activity. The system can also be used for enzymes having water soluble substrates, by contacting the enzyme-containing membrane with substrate dissolved in an aqueous phase. Moreover, the system can be used to react substances in aqueous media over a wide pH range, because the organic liquid insulates the membrane from the pH of the surrounding aqueous medium.

The invention also can be used to provide sustained

controlled release of active enzyme over time, as described more fully below.

The Enzyme System

Enzymes used in the above-described system may be virtually any known enzyme, but preferred enzymes are those catalyzing reaction of a lipophilic substance, i.e., one that prefers non-polar, hydrophobic water-immiscible liquids over polar liquids such as water; i.e. the substance should be sparingly (if at all) soluble in water, but easily dissolved in the water-immiscible organic liquid chosen.

The organic liquid or solvent used could be any apolar organic solvent which is immiscible with water. In general, the solubility of water in these organic solvents solvents should be less than 10% and preferably less than 1% by weight. The dipole moment of these

solvents in general will be between 0 and 2.5 and preferable under 1 Debye unit. The dielectric constants of these solvents in general will be under 20 and preferably under 10. It will be preferable to have solvents with relatively high boiling points specifically for applications invoving higher temperatures. Examples of suitable solvents in this class include toluene, benzene, xylene, hydrocarbons, oils, higher alcohols etc. The organic liquid can be readily selected for a given enzyme as described herein. The final organic liquid content of the membrane should be over 90% of the total liquid in the membranes and preferable over 98% of the total liquid.

The preferred carriers are membranes, although other forms of carrier could be used. Specifically, the membrane can be in any convenient form such as flat

sheet, hollow fibers, tubes, sponges or even porous rods or fabricated forms from basic membrane structures. Membranes with larger pores in the range of 10 Angstroms - 100 micron and/or initial void volumes between 20-97% of wet volume are suitable for these procedures, with pore sizes in 10 Angstroms - 5 micron and/or initial void volumes between 50-90% being the most preferred range.

The two primary requirements of membranes for such a procedure are that: i) the initial pore sizes of the membranes are large enough to allow the enzymes of interest to enter the membrane matrix by diffusion (in the case of asymmetric membranes, at least the pores on the more open spongy side are large enough for the enzymes to diffuse into the membrane matrix); and ii) the water (or other non-solvent) in the pores is in its

non-equilibrium state, such that the pores can be collapsed irreversibly upon drying. These requirements can be easily met by membranes synthesized from a wide variety of relatively hydrophobic polymers or their derivitives, as described below. Also the membrane should be resistant to the organic solvent of interest.

As noted, the initial water (non-solvent) content should be such that, upon drying or evaporation of the non-solvent, the pores will collapse irreversibly. This requirement is easily met by membranes fabricated from a wide variety of relatively hydrophobic polymers, copolymers or their derivatives such as nylons, polyesters, polysulfones, polyacrylonitriles, polycarbonates, polyvinylchlorides, cellulose esters etc. When solutions of these membranes are cast, spun or extruded and allowed to coagulate in a non-solvent bath or in

atmospheric humidity, the membranes contain a high fraction of non-solvent, but upon drying the pore-structure generally collapse irreversibly such that they will not regain the original amount of water or non-solvent upon rewetting.

Alternatively, the membrane could be hydrophilic such that the pores would collapse and the membranes would deswell when water or hydrophilic solvent is replaced by non-polar solvents. Such requirements are also easily met by a number of common polymers such as poly-HEMA or their derivatives, cellulosic or their derivatives, hydrolyzed polyacrylonitrile or their derivatives, collagen, polyvinyl alcohol of their derivatives, etc.

The choice of hydrophobic or hydrophilic polymer matrices depends upon the application. In the case of

hydrophilic membranes, the entrapment as described above will be due to the desolvation and consequent partial collapse of the membrane structure in presence of the organic liquids. When such membranes are used in contact with aqueous liquids, e.g., as in diagnostic strips, the membranes would gradually reswell and water will preferentially displace the entrapped organic liquids and cause some of the immobilized enzymes to leach into the analate solution. Such loss would be of little consequence in the case of disposal diagnostic strips but is not desirable for applications requiring continuous processes as for example in bioreactors.

For continuous processes conducted in contact with aqueous or hydrophilic media, hydrophobic membrane matrices which undergo permanent shrinkage of pores

will be more desirable. The hydrophilic membrane matrices, however are preferred for controlled release of enzymes, because the enzymes would be stable for a long time but could be released slowly into external aqueous media when contacted by such media. Such systems are useful for example in enzymatic processes involving in situ clean up of soil or water contamination.

Enzyme stabilization in hydrophilic matrices can also be used as a storage device to keep the enzymes active until needed. This would be particularly useful for in-field applications where it may not be practicable to have refrigeration. At the time of use, stabilized enzymes trapped in hydrophilic matrices can release the enzymes into outside aqueous solution. Such stabilized enzymes therefore can also be used

with applications requiring hydrophilic substrates and/ or cofactors, both of which are largely insoluble in the organic liquids.

Manufacture

One convenient way to manufacture these stabilized membranes under mild conditions (shown in Fig. 1) is to start with a suitable synthetic membrane containing water or aqueous buffer solution in its pores and with pore-sizes large enough to accomodate the enzyme molecules. The membrane is next placed in a buffered solution of enzyme at its optimum pH and the enzyme is allowed to diffuse into the membrane matrix. After the diffusional exchange, the membrane is partially dried either by gently squeezing the membrane between paper towels or by subjecting it to vacuum. The membrane is of such a type that this partial drying

causes partial collapse of the pores, thereby effectively entrapping the enzyme molecules within the pores.

After the entrapment step, the membrane is next placed in a water miscible organic solvent such as acetone, ethanol, etc., and the water in the membrane is exchanged via diffusional exchange. Once this exchange is complete, the enzyme-entrapped membrane is further placed into the desired organic liquid. The diffusional exchange causes the enzyme molecules to be surrounded by the appropriate organic liquids. Since the enzymes are effectively trapped within the pores or void spaces of the polymer matrix, they do remain well-dispersed during the diffusional exchange. The hydrophilicity of parts of the enzyme will result in a small amount of bound water enveloped around the enzyme molecules. Based on current theoretical

understanding, it appears that the bound water around enzyme molecules ensures their normal functioning, and the hydrophobic medium surrounding them establishes hydrophobic interaction to prevent unfolding of the enzyme molecules.

Although the above method is a gentle and straightforward method for making such membranes, there are a variety of other common methods of immobilization which are familiar to those in the field, including cross-linking and entrapment, covalent binding and entrapment, etc. These methods generally could be used for the present invention, but the particular method chosen should be selected to avoid damage to the enzymes. The membrane pores or void spaces should be filled with appropriate organic solvent as soon as possible, subsequent to immobilization.

Finally, even though the present invention describes stabilization of enzymes which are isolated and not a part of living or dead cells, it is possible to prepare such membranes from cell fragments or even whole cells without isolating the enzymes from the cells, The use of the invention is not limited by whether the enzymes are in isolated and purified form or part of cell fragments and therefore in crude, unpurified forms.

The following examples illustrate the invention and they are not to be construed as limitations on it.

Example 1:

Microporous membranes were made by dissolving polyacrylonitrile (molecular weight 150,000)in DMSO at a concentration of 6% by weight, and the solution was filtered through 5 micron stainless steel wire-mesh. The filtered solution was cast on a glass plate with a casting knife resulting in a 10 mil (250) thick solution layer and allowed to coagulate at 70° F and between 70-75% humidity. The polymer solution coagulated in 2-3 hours, giving a microporous membrane with maximum pore-size of 0.8 microns as judged by bubble point. The water content of this membrane was 94% by weight.

The membrane was washed with water for several days and punched into discs of 25 mm diam-